UNIVERSITI PUTRA MALAYSIA

ENANTIOSELECTIVE ESTERIFICATION OF (±)-MENTHOL WITH BUTYRIC ANHYDRIDE BY CHEMICALLY MODIFIED *CANDIDA RUGOSA* LIPASE

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By

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ENANTIOSELECTIVE ESTERIFICATION OF (±)-MENTHOL WITH BUTYRIC ANHYDRIDE BY CHEMICALLY MODIFIED *CANDIDA RUGOSA* LIPASE

By

HALILA BINTI JASMANI

May 2003

Chairman : Professor Abu Bakar Salleh, Ph.D.

Faculty : Science and Environmental Studies

Commercial lipase from *Candida rugosa* was chemically modified with the aim to improve its catalytic properties in organic solvents. The chemical modifiers, aldehydes and monomethoxy polyethylene glycols, were covalently linked to the lysine residues at the surface of the enzyme. Enzymatic enantioselective esterification of racemic menthol in organic solvents using butyric anhydride as acylating agent was performed with the chemically modified lipases. Different degrees of modification, organic solvents, reaction temperatures and water activity were examined for the influence on the percent yield and enantioselective formation of (-)-menthyl butyrate. The percent yield increased as the degree of modification increased but decreased slightly for the highest degree of modification. Organic solvents with log P values above 2.5 gave higher yield, however high enantioselectivity was obtained in all the organic solvents tested. The enantioselectivity towards (-)-menthol decreased considerably as the reaction temperature was increased. Enzyme derivatives exhibited better activity and enantioselectivity at high a_w. The alkylated lipases showed higher thermal, solvent

and storage stability than PEG-lipases. Propionyl-lipase in particular was highly thermostable in *iso*octane.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi syarat untuk mendapatkan Ijazah Doktor Falsafah

PEGESTERAN PEMILIHAN ENANTIO (±)-MENTOL DAN BUTIRIK ANHIDRIDA OLEH LIPASE *CANDIDA RUGOSA* TERUBAHSUAI KIMIA

Oleh

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Mei 2003

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Fakulti : Sains dan Pengajian Alam Sekitar

Lipase komersial dari *Candida rugosa* telah diubahsuai secara kimia dengan tujuan untuk memperbaiki sifat pemangkin dalam pelarut organik. Pengubahsuai kimia, aldehid dan monometoksi polietilena glikol diikat secara kovalen dengan residu lisin pada permukaan enzim. Pengesteran pilihan enantio secara enzim bagi mentol rasemik dalam pelarut organik menggunakan butrik anhidrid sebagai agen pengasilan telah dilakukan menggunakan lipase terubahsuai secara kimia. Darjah ubahsuaian, pelarut organik, suhu tindakbalas dan akitiviti air yang berlainan telah dikaji bagi kesan keatas peratus hasilan dan pembentukan pilihan enantio (-)-mentil butrat. Peratus hasilan meningkat dengan peningkatan darjah ubasuaian tetapi menurun sedikit bagi darjah ubahsuaian tertinggi. Pelarut organik bernilai log P 2.5 keatas memberikan hasilan tinggi, walaubagaimanapun pemilihan enantio tinggi dalam semua pelarut organik yang diuji. Pemilihan enantio keatas (-)-mentol menurun ketara bila suhu tindakbalas meningkat. Enzim terbitan mempamirkan aktiviti dan pemilihan enantio lebih baik pada a_w tinggi. Lipase teralkil menunjukkan kestabilan therma, pelarut dan penyimpanan lebih tinggi dari lipase-PEG. Lipase-propionil terutamanya mempunyai kestabilan terma yang tinggi dalam *iso*oktana.

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I certify that an Examination Committee met on 16th May 2003 to conduct the final examination of Halila binti Jasmani on her Doctor of Philosophy thesis entitled "Enantioselective Esterification of (±)-Menthol with Butyric Anhydride by Chemically Modified *Candida rugosa* Lipase" in the accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981 The Committee recommends that the candidate be awarded the relevant degree Members of the Examination Committee are as follow

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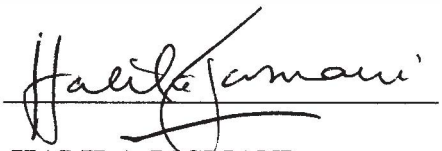
DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.



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CHAPTER I

INTRODUCTION

Lipase-catalyzed reactions in organic media become a field of increasing interest in recent years (Ghandi, 1997; Benjamin and Pandey, 1998 and Jaeger and Reetz, 1998). Products of lipase-catalyzed reactions find widespread applications in industries such as their addition to detergents, the production of food ingredients, pharmaceuticals, cosmetics, perfumery and other organic synthetic materials. However, one of the drawbacks for industrial application of enzymes is the relatively easy deactivation of enzymes when they are subjected to heat, extreme pH or proteases. Increasing enzyme thermostability would allow enzymatic reactions to be carried out at higher temperatures; this would help to increase conversion rates and substrates' solubility and to reduce the possibility of microbial growth and the viscosity of the reaction medium. Strategies that have been proposed include immobilization (Monsan and Combes, 1988), protein engineering (Gupta, 1991) and chemical modification (Mozhaev *et al*., 1992). The latter appears to be the most popular approach, taking into account the amount of research and the significance of the results obtained.

Lipases are considered to be the most versatile group of enzymes; they undergo simple reactions and do not require cofactors. Furthermore, they possess a broad substrate specificity and exhibit high enantioselectivity. It is generally accepted that when enzymes are placed in organic media they exhibit altered properties such as an enhanced thermostability and favourable thermodynamic equilibrium shift for many reactions. In addition, industrial utility of non-aqueous enzymatic reactions is

enhanced because of an increased solubility of the substrates, ease of product and enzyme recovery and a reduced risk of microbial contamination of reactors.

Lipases catalyze esterification and interesterification reactions in organic media. *Candida rugosa* lipase is of a particular interest since it is commercially available and has been widely used in biotransformations due to its high activity both in hydrolysis and synthesis reactions. Although lipases are increasingly being used in synthetic organic reactions, their catalytic properties are not always optimal. Therefore it is possible to redesign the functional and physical properties of lipases so that they have the desired properties. Protein surface characteristics, hydrophobicity/hydrophilicity balance and charge distribution seemed to play important roles in the catalytic activity. It is a possibility to change the biocatalyst activity by modifying the enzyme microenvironment. Different approaches have been applied to modify lipases to produce beneficial properties such as to increase activity, stability and solubility.

One of the strategies that have been reported by chemically modifying the enzyme is covalent attachment to the lysine residues on the protein surface to a modifier. Modifiers that have been used for this purpose include amphiphilic polymers such as monomethoxy polyethylene glycol (Basri *et al*., 1995 and Hernaiz *et al*., 1997), hydrophobic imidoesters (Basri *et al*., 1992) and aldehydes (Salleh *et al*., 1990; Ampon *et al*., 1991 and Ampon *et al*., 1992). However, other methodologies have also been described. Among them include surfactant coated lipase (Goto *et al*., 1994), treating with short-chain polar organic solvents (Chamorro *et al*., 1998), TPI (trapping in the presence of interface) (Gonzalez-Navarro and Braco, 1998), the use

of natural fatty acids as amphiphilic modifier (Fishman *et al.*, 1998) and replacing the lactose used in the commercial preparation of lipase by dextrans (de la Casa *et al.*, 1999). In general, these modifications increase the activity in the organic media compared to the unmodified enzyme. Gonzalez-Navarro and Braco (1998) and Chamorro *et al.* (1998) attributed the enhanced activities due to the lipase having attained the open activated conformation.

Enantioselectivity is defined as the preferential formation of enantiomer of the product over another, or the preferential reaction of one enantiomer of the (usually racemic) starting material over the other. Enantiopure materials are important for synthesizing natural products, which are always single enantiomers. Methods of synthesizing other products that lead to a single enantiomer as opposed to a racemate are becoming increasingly important. Enantioselectivity is perhaps the most attractive feature of enzyme-catalyzed synthesis. The application of enzymes in the synthesis of optically pure compounds is becoming increasingly prominent (Schoffers *et al.*, 1996).

The active components of many drugs and medicines are frequently chiral molecules and it is likely that in many countries legislation will soon require these components to be used as single enantiomers. This is because drugs interact with enzyme systems and receptors and one enantiomer of the drug is usually more active than the other. Sometimes one enantiomer may be completely inactive; in other cases it may lead to undesirable side effects. The racemic mixtures of non-steroidal anti-inflammatory drugs (NSAIDS) such as ibuprofen, ketoprofen and naproxen are still being used although the (*S*)-enantiomers are the therapeutically important